Helicobacter pylori FlhB function: the F1hB C-terminal homologue HP1575 acts as a "Spare part" to permit flagellar export when the HP0770 FlhB(CC) domain is deleted

JOURNAL OF BACTERIOLOGY, Nov. 2006, p. 7531–7541 Vol. 188, No. 21
0021-9193/06/$08.00+0 doi:10.1128/JB.00263-06

Helicobacter pylori FlhB Function: the FlhB C-Terminal Homologue HP1575 Acts as a "Spare Part" To Permit Flagellar Export When the HP0770 $FlhB_{CC}$ Domain Is Deleted†

Matthew E. Wand,[1]‡ R. Elizabeth Sockett,[2] Katy J. Evans,[2] Neil Doherty,[1] Paul M. Sharp,[2] Kim R. Hardie,[3] and Klaus Winzer[3]*

Institute of Infection, Immunity and Inflammation, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom[1]; Institute of Genetics, School of Biology, Medical School, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom[2]; and Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham NG7 2RD, United Kingdom[3]

Received 20 February 2006/Accepted 1 August 2006

In *Helicobacter pylori* 26695, a gene annotated HP1575 encodes a putative protein of unknown function which shows significant similarity to part of the C-terminal domain of the flagellar export protein FlhB. In *Salmonella enterica*, this part ($FlhB_{CC}$) is proteolytically cleaved from the full-length FlhB, a processing event that is required for flagellar protein export and, thus, motility. The role of FlhB (HP0770) and its C-terminal homologue HP1575 was studied in *H. pylori* using a range of nonpolar deletion mutants defective in HP1575, HP0770, and the CC domain of HP0770 ($HP0770_{CC}$). Deletion of HP0770 abolished swimming motility, whereas mutants carrying a deletion of either HP1575 or $HP0770_{CC}$ retained their ability to swim. An *H. pylori* strain containing deletions in both HP1575 and $HP0770_{CC}$ was nonmotile and did not produce flagella, suggesting that at least one of the two proteins had to be present for flagellar assembly to occur. Indeed, motility was restored when HP1575 was reintroduced into this strain immediately downstream of, but not fused to, the truncated HP0770 gene. Thus, HP1575 can functionally replace $HP0770_{CC}$ in this background. Like FlhB in *S. enterica*, HP0770 appeared to be proteolytically processed at a conserved NPTH processing site. However, mutation of the proline contained within the NPTH site of HP0770 did not affect motility and flagellar assembly, although it clearly interfered with processing when the protein was heterologously produced in *Escherichia coli*.

The gastric pathogen *Helicobacter pylori* is a major causative agent of chronic superficial gastritis and peptic ulcer disease and, more seriously, has an important role to play in the development of adenocarcinoma of the distal stomach in humans (18, 35). For many pathogenic gut bacteria, flagellum-dependent motility and chemotaxis are important factors in the colonization process of a potential host and the establishment of a successful infection. In *H. pylori* flagellar motility is essential for colonization of gnotobiotic piglets (4) and mice (3).

H. pylori cells normally possess a unipolar bundle of two to six sheathed flagella. Each flagellum is about 3 μm long and shows a typical bulb-like structure at its distal end that represents a dilation of the flagellar sheath. The sheath is continuous with the outer membrane and contains lipopolysaccharide and protein; its function is protection of the acid-labile flagellar structure from stomach acid (11). Aside from the appearance of a sheath, *H. pylori* flagella are very similar to those of enteric bacteria. The *H. pylori* flagella are composed of three structural elements, which are the membrane-bound basal body, the hook, and the flagellar filament (29). Many of the enteric bacterial flagellar proteins have homologues in *H. pylori*, although its flagellar apparatus appears to differ slightly from the well-characterized *Escherichia coli* and *Salmonella enterica* serovar Typhimurium flagellar paradigms; e.g., *H. pylori* contains two copies of *flgE* both of which encode hook subunits that are larger than their *E. coli* counterpart. This feature may be related to the physics of terminal flagellum rotation or motility in a viscous mucosal environment (17). Flagellar assembly in *H. pylori* appears to occur in a manner similar to that described for enteric bacteria (5, 22, 23): the export apparatus is assembled from proteins encoded by *fliH*, *fliI*, *fliJ*, *fliO*, *fliP*, *fliQ*, *fliR*, *flhA*, and *flhB*, and the exported flagellar proteins (such as hook, hook-capping protein, and flagellins) are then transported down the central pore in the growing flagellar structure.

FlhB is a protein with an important role in flagellum assembly. In various enterobacteria, including *S. enterica* serovar Typhimurium (21) and *Yersinia enterocolitica* (7), *flhB* is present within an operon that also contains genes for FlhA and FlhE. The *flhB* and *flhA* genes encode highly hydrophobic polypeptides with molecular masses in *Salmonella* of 42 and 75 kDa, respectively, (21). Both proteins have several potential membrane-spanning segments, suggesting that they are integral membrane proteins. FlhB resides in the central pore of the basal body complex in close association with FlhA, and its

* Corresponding author. Mailing address: Institute of Infection, Immunity, and Inflammation, University of Nottingham, Centre for Biomolecular Sciences, Nottingham, NG7 2RD, United Kingdom. Phone: 44 115 84 67954. Fax: 44 115 84 67951. E-mail: Klaus.Winzer@nottingham.ac.uk.

‡ Present address: Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford, OX1 3SY, United Kingdom.

† Supplemental material for this article may be found at http://jb.asm.org/.

function is linked to flagellar export. Early studies by Hirano et al. suggested a role in hook length regulation (13). Later, FlhB was shown to act as a gating mechanism to prevent the incorrect type of flagellar protein from being exported into the growing flagellar structure (24). During the flagellar assembly process, the export apparatus switches from rod- and hook-type proteins to filament and filament cap proteins. Macnab and coworkers have studied the role of FlhB in substrate specificity switching in *S. enterica* serovar Typhimurium in great detail (10, 24, 39, 41). Western blot analysis of FlhBs with six-histidine tags fused to either the C- or N-terminal ends showed the presence of two forms of the protein: processed and unprocessed (24). Subsequent N-terminal sequencing indicated that there is a highly sensitive cleavage site between amino acids Asn^{269} and Pro^{270} in the cytoplasmic domain of FlhB. Cleavage of FlhB at this site is a requirement for flagellar protein export and, thus, flagellum assembly (24). It has been proposed that the function of this processing, which generates two proteins, the FlhB fragment containing the transmembrane helices and the N-terminal part of the C-terminal domain ($FlhB_{TM+CN}$) and the FlhB fragment containing the CC domain ($FlhB_{CC}$), is to change the substrate specificity for flagellar export from rod- and hook-type proteins to filament-type proteins (10). In *Salmonella* the function of FlhB requires interaction of the cleaved $FlhB_{CC}$ domain with the hook length control protein FliK (13, 24), a functional homologue of which has recently been identified in *H. pylori* (32). Cleavage has also been investigated in *Yersinia pseudotuberculosis* where YscU, a FlhB homologue which is part of a type III secretion system, is cleaved between Asn^{263} and Pro^{264} (16). Interestingly, mutations within the conserved processing site, although abolishing protein cleavage, did not interfere with Yop secretion (16). However, overexpression of these YscU variants resulted in severe growth inhibition, suggesting that YscU cleavage is required to maintain a nontoxic fold. In some bacteria, mutation of *flhB* resulted in phenotypes not directly associated with motility. For instance, in *Pseudomonas putida* FlhB appears to be involved in solvent tolerance (34), and in *Campylobacter jejuni*, *flhB* inactivation influenced cell shape (19).

Previous work has provided evidence to suggest that an *flhB* mutant of *H. pylori* is nonmotile, like its *Salmonella* counterpart (9). Motility analysis using soft agar and electron microscopy showed that colonies did not swarm and that cells lacked flagella. Colonization studies in mice revealed that mice infected with an FlhB (HP0770) null SS1 strain showed no colonization at either 2 or 8 weeks after initial infection, supporting the link between colonization and flagellar motility (9).

In *H. pylori*, a gene present at an unrelated site on the chromosome, HP1575, encodes an approximately 12-kDa protein that shows significant sequence similarity to the proposed $FlhB_{CC}$ domain of the *H. pylori* FlhB protein (the *H. pylori* FlhB protein will be referred to as HP0770 in the remainder of this study to distinguish it from homologues found in other organisms) (Fig. 1A). The protein encoded by HP1575 is (highly) hydrophilic, reflecting its homology to the soluble cytoplasmic domain of FlhB, but the surrounding genes in the putative operon of HP1575 appear to have no flagellum-related role. HP1575 has been annotated as part of a putative ABC transporter, and the gene has been named *abcB* (12; www.tigr.org). This is mainly due to the strong similarity of the gene immediately downstream of HP1575, namely *abcC*, to the *nikD* gene of an ATP-dependent nickel ABC-type transporter (12). However, HP1575 and its putative homologues in other organisms have not yet been studied experimentally. Here, we analyze the HP1575 gene of *H. pylori* and show that it has a role in flagellar biogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *H. pylori* was routinely grown and maintained on Columbia blood base agar (no. 2, with 5% [vol/vol] horse blood; Oxoid) plates. *H. pylori* was cultured at 37°C for 24 to 48 h as required in a variable atmosphere incubator (Don Whitley Scientific) in an atmosphere of 5% CO_2, 86% N_2, and 6% O_2 (all, vol/vol). Analysis of *H. pylori* swimming motility was performed in brain heart infusion medium (Oxoid) at a pH of 6.0, supplemented with 10% (vol/vol) fetal bovine serum (Gibco), and 0.35% (wt/vol) Bacto agar (BD Biosciences) (referred to as motility agar). Excess moisture was carefully dried from the agar surface before use and after inoculation; agar plates were incubated at 37°C for up to 5 days in the variable atmosphere incubator cabinet. *E. coli* strains were maintained on Luria-Bertani (LB) agar or in LB broth.

Growth media were supplemented with the following antibiotics as required: ampicillin at 100 μg/ml, chloramphenicol at 30 μg/ml, and kanamycin at 50 μg/ml.

Molecular techniques. Primers used in this study are listed in Table 2. Preparation of plasmid DNA, restriction enzyme digests, ligation, separation of fragments by gel electrophoresis, and transformation of *E. coli* strains were performed according to standard methods (33). DNA fragments were purified from agarose gels using a QIAquick gel extraction kit (QIAGEN, United Kingdom) according to the manufacturer's instructions. *H. pylori* genomic DNA was isolated using a modification of the guanidium lysis protocol of Pitcher and coworkers (30). All plasmid constructs were verified using PCR, restriction digestion, and sequencing. A schematic showing the representation of the HP0770 and HP1575 gene regions for all mutants constructed and used in this study is shown in Fig. 2.

Transformation of *H. pylori* strains. Disrupted or otherwise mutated HP0770 and HP1575 genes were introduced into *H. pylori* and integrated into the chromosome by natural transformation, allelic exchange, and antibiotic rescue. Wild-type recipient strains were assessed for motility prior to transformation by methods already described (see above). Positive strains were selected for by kanamycin or chloramphenicol selection and verified by PCR using primers that were designed to *Helicobacter* DNA sequence outside possible mutant recombination sites. Specific integration was also verified by nucleotide sequencing across the points of insertion using the same primers.

Construction of an *H. pylori* HP1575 deletion strain. The putative *H. pylori* 273-bp HP1575 gene was amplified from *H. pylori* 26695 genomic DNA by PCR, using forward and reverse oligonucleotide primers (HP1575UF/HP1575DR) that were designed to amplify the HP1575 gene with approximately 500 bp of upstream and downstream flanking sequence; the product was cloned into pGEM-Teasy to produce plasmid pMWA1. A deletion mutation of HP1575 was generated by inverse PCR mutagenesis with the primer pair HP1575REGKUF and HP1575REGKDR using pMWA1 as a template. A 900-bp fragment from pUC18K2 (20) containing a gene encoding resistance to kanamycin (*aphA-3*) (38) or a 700-bp nonpolar derivative of the chloramphenicol resistance gene (*catB3*) (40) from pBSC103 was cloned into pMWA1L to generate pMWA2 and pMWA6, respectively, which were transformed into recipient wild-type *H. pylori* strain J99 with positive selection for kanamycin or chloramphenicol as required. Resultant recombinant strains J99ΔHP1575::Km and J99ΔHP1575::Cm carried an in-frame deletion of amino acids 11 to 74, inclusive, of HP1575 (Fig. 2, B).

Construction of an *H. pylori* HP0770 deletion strain. Plasmid pMW2 containing an *aphA-3* (Km^r)-interrupted HP0770 gene was constructed in the standard cloning vector pSP72 by a three-step process. Oligonucleotide primers were designed to amplify two fragments from strain 26695 chromosomal DNA. Fragment 1 (approximately 590 bp) contained the 5′ end, and fragment 2 (approximately 550 bp) contained the 3′ end of the HP0770 gene. Fragment 1 was cloned into pSP72 vector to form plasmid pMW2F1 (step 1). The nonpolar kanamycin cassette was cloned into pMW2F1 to produce plasmid pMW2F1::Km (step 2). Fragment 2 was then introduced into plasmid pMW2F1::Km to generate the recombinant plasmid pMW2 (step 3). This plasmid was transformed into motile recipient strains. Transformation of the wild-type strain J99 generated the re-

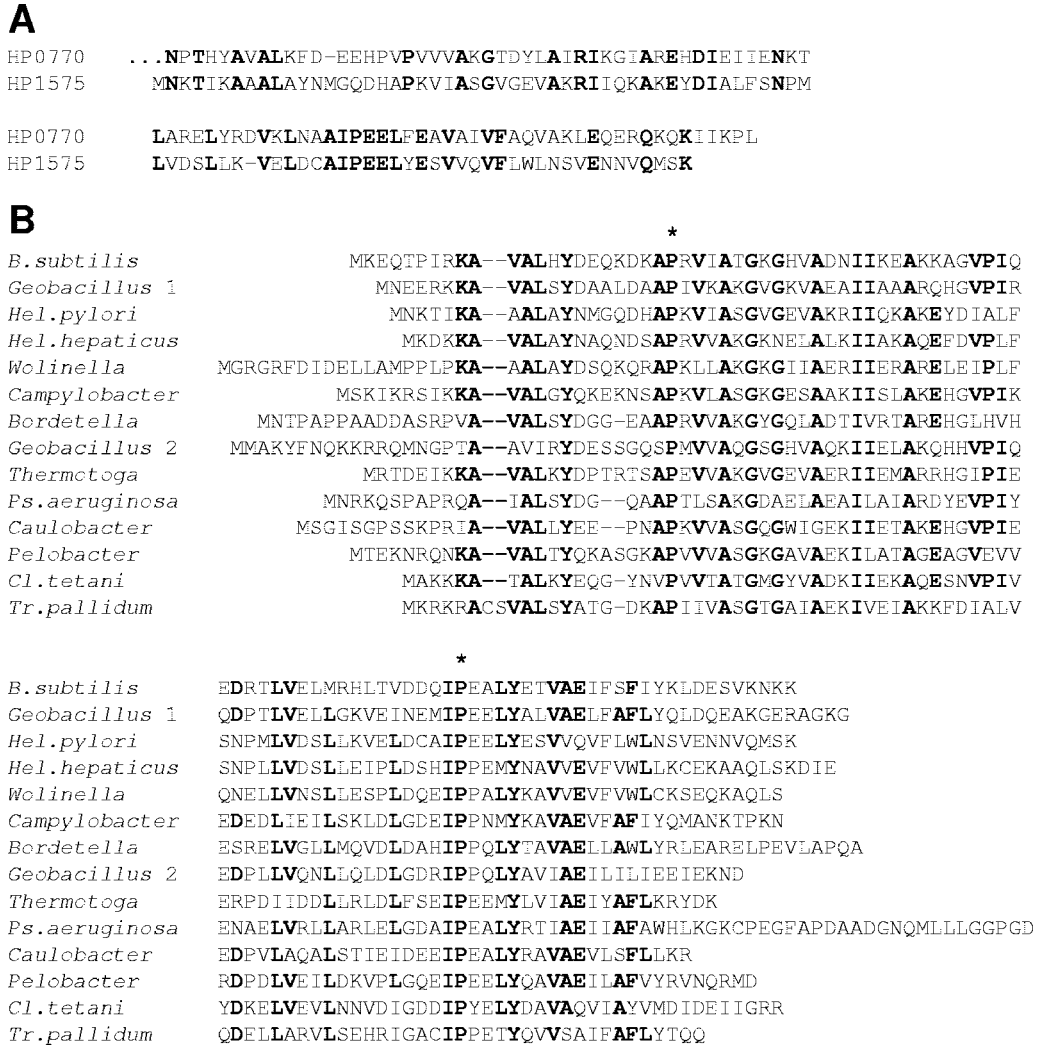

FIG. 1. HP1575 has similarity to $FlhB_{CC}$, with homologues encoded by numerous bacteria. (A) Alignment of HP1575 (FlhB2) and the C-terminal end of HP0770 (FlhB). The cleavage site for $FlhB_{CC}$ is underlined. Identical residues in the two sequences are in bold. (B) Alignment of 14 representative HP1575 (FlhB2) homologues from a range of species, including the two copies from *Geobacillus kaustophilus*. Residues conserved across more than 50% of the sequences are shown in bold. Two Pro residues conserved among all sequences (including those not shown here) are indicated by as asterisk above the alignment. The abbreviated genus names are as follows: B, *Bacillus*; Hel. *Helicobacter*; Ps, *Pseudomonas*; Cl, *Clostridium*; and Tr, *Treponema*. Full details of the species names and accession numbers for the sequences are available from the authors on request.

combinant strain J99ΔHP0770::Km which carried an in-frame deletion of HP0770 from amino acids 13 to 347, inclusive (Fig. 2, C).

Construction of an *H. pylori* $HP0770_{CC}$ deletion strain. Primers (HP0770MF/HP0770NDR) were designed to amplify a 1.4-kb fragment comprising the HP0770 upstream region and the exact $HP0770_{TM+CN}$ part of the HP0770 gene (in agreement with the terminology introduced by Fraser et al. [10] for *S. enterica* serovar Typhimurium *flhB*, this part of the gene encodes the transmembrane region as well as the N-terminal part of the C-terminal domain of HP0770). This PCR also introduced a stop codon in HP0770 after asparagine 265, thus ensuring exact translation of the encoding polypeptide. The 590-bp HP0770 upstream region was excised from plasmid pMW2 and replaced with the new 1.4-kb fragment to create plasmid pMW3. Transformation of the wild-type strain J99 with this plasmid generated the recombinant strain $J99\Delta HP0770_{CC}$::Km that contained a deletion of HP0770 from amino acid 265 onwards (Fig. 2D).

Construction of an *H. pylori* HP0770(P266G) strain. Forward and reverse oligonucleotide primers FlhBU/FlhBD were designed to amplify the HP0770 gene with an N-terminal His-tag. The PCR product was ligated into the inducible plasmid pFLAG-CTC (Sigma) to produce plasmid pFLAGMW1 that contained HP0770 with an N-terminal His tag and a C-terminal FLAG tag. An HP0770 gene containing a mutation in the NPTH processing site of the encoded protein was generated by the introduction of a point mutation that altered the Pro^{266} residue to Gly^{266}, thus creating a processing site that read NGTH in a full-length HP0770 gene that encoded a protein with an N-terminal His tag and a C-terminal FLAG tag in plasmid pFLAGMW3. The inserts of pFLAGMW1 and pFLAGMW3 were removed and inserted into pMW2 (where they replaced the original HP0770 upstream region) to generate plasmids pMW4 and pMW5, respectively. Transformation of the wild-type strain J99 with these plasmids generated the recombinant strains J99THP0770-Km and J99THP0770(P266G)-Km, respectively (designed to produce FLAG-tagged HP0770).

Construction of double mutants. The plasmid constructs described above encoding the mutated HP0770 gene were used to transform J99ΔHP1575::Cm, thus generating the double mutants listed in Table 1.

Complementation of mutants. Single gene complementation tests of the double $\Delta HP0770_{CC}\Delta HP1575$ mutant were performed by the reintroduction of either HP1575 or $HP0770_{CC}$ immediately upstream of the *aphA-3* (Km^r) cassette (Fig. 2, F). To do this $HP0770_{CC}$ and HP1575 were amplified by PCR using primers HP0770FCP/HP0770KpnIGR and HP1575FCP/HP1575KpnIGR, respectively, and introduced into plasmid pMW3 to create pMW3a (HP1575) and pMW3b

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
H. pylori strains		
26695	Wild-type sequence strain	37
J99	Wild-type motile strain	1
J99ΔHP1575::Km	J99 derivative; ΔHP1575 Ala^{11} to Val^{74}; Km^r	This study
J99ΔHP1575::Cm	J99 derivative; ΔHP1575 Ala^{11} to Val^{74}; Cm^r	This study
J99ΔHP0770::Km	J99 derivative; ΔHP0770 Lys^{13} to Glu^{347}; Km^r	This study
J99ΔHP0770$_{CC}$::Km	J99 derivative; ΔHP0770$_{CC}$ Pro^{266} to Leu^{358}; Km^r	This study
J99ΔHP0770ΔHP1575	J99 derivative; ΔHP0770 Lys^{13} to Glu^{347}; ΔHP1575 Ala^{11} to Val^{74}; Cm^r Km^r	This study
J99ΔHP0770$_{CC\Delta}$HP1575	J99 derivative; ΔHP0770$_{CC}$ Pro^{266} to Leu^{358}; ΔHP1575 Ala^{11} to Val^{74}; Cm^r Km^r	This study
J99ΔHP1575HP1575Comp	J99 derivative; ΔHP1575 Ala^{11} to Val^{74}; ΔHP0770$_{CC}$ Pro^{266} to Leu^{358} with independently transcribed in *cis* HP1575 immediately downstream; Cm^r Km^r	This study
J99ΔHP1575HP0770$_{CC}$Comp	J99 derivative; ΔHP1575 Ala^{11} to Val^{74}; ΔHP0770$_{CC}$ Pro^{266} to Leu^{358} with independently transcribed in *cis* HP0770$_{CC}$ immediately downstream; Cm^r Km^r	This study
J99THP0770-Km	J99 derivative; C-terminal FLAG-tagged HP0770; Km^r	This study
J99THP0770(P266)G-Km	J99 derivative C-terminal FLAG-tagged HP0770 with single point mutation Pro^{266} to Gly^{266}; Km^r	This study
E. coli strains		
DH5α	λ^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	42
RP437	*thr-1*(Am) *leuB6 his-4 metF159*(Am) *eda-50 rpsL136 thi-1 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78*	28
Plasmids		
pFLAG-CTC	f1 *ori*, pBR322 *ori*; Amp^r; cloning vector; adds C-terminal FLAG tag	Sigma
pSP72	f1 *ori*, ColE1 *ori*; Amp^r; cloning vector	Promega
pUC18K2	ColE1 *ori*; Amp^r Km^r; source of *aphA-3* nonpolar cassette	20
pBSC103	f1 *ori*, pUC *ori*, Amp^r Cm^r; source of chloramphenicol cassette	40
pMW2	Amp^r Km^r; 1.1-kb HP0770 deletion by insertion of 0.9-kb *aphA-3* cassette into EcoRI and BamHI sites	This study
pMW3	pMW2 derivative; Amp^r Km^r; 0.6-kb HP0770 deletion region replaced by 1.4-kb ΔHP0770$_{CC}$ using ClaI and EcoRI sites	This study
pMW4	pMW2 derivative; Amp^r Km^r; 0.6-kb HP0770 deletion region replaced by full-length 1.7-kb HP0770 C-terminally FLAG-tagged HP0770 using ClaI and EcoRI sites	This study
pMW5	pMW2 derivative; Amp^r Km^r; 0.6-kb HP0770 deletion region replaced by full-length 1.7-kb C-terminally FLAG-tagged HP0770 with single point mutation (Pro^{266} changed to Gly^{266}) using ClaI and EcoRI sites	This study
pMW3a	pMW3 derivative; Amp^r Km^r; full-length 0.27-kb HP1575 cloned into KpnI and EcoRI sites	This study
pMW3b	pMW3 derivative; Amp^r Km^r; full-length 0.27-kb HP0770$_{CC}$ cloned into KpnI and EcoRI sites	This study
pFLAGMW1	Amp^r; full-length 1.1-kb N-terminally His-tagged HP0770 cloned into HindIII and SalI sites of pFLAG-CTC; the encoded protein is N-terminally His and C-terminally FLAG tagged	This study
pFLAGMW3	pFLAGMW1 derivative; Amp^r; full-length 1.1-kb N-terminally His and C-terminally FLAG-tagged HP0770 with single point mutation changing Pro^{266} to Gly^{266}	This study
pMWA1	Amp^r; full length 1.3 kb HP1575 and surrounding region cloned into NcoI and SalI sites	This study
pMWA6	Amp^r Km^r; 0.27-kb HP1575 deleted and replaced by 0.7-kb *cat* cassette using EcoRI and BamHI sites	This study

(HP0770$_{CC}$). Transformation of *H. pylori* J99ΔHP1575::Cm and subsequent recombination into the chromosome yielded strains J99ΔHP1575HP1575Comp and J99ΔHP1575HP0770$_{CC}$Comp, respectively.

Immunoblotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) and transferred to nitrocellulose membrane (Boehringer Mannheim) using a Mini Trans-Blot transfer cell (Bio-Rad) containing 10 mM Tris, 100 mM glycine, and 20% (vol/vol) methanol. The membrane was stained with Ponceau S (2%, vol/vol) solution of 3-hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo) phenylazo]-2,7-naphthalenedisulfonic acid (Sigma) for 10 min at room temperature. Excess Ponceau S stain was removed using distilled water, and the position of markers was noted. The membrane was blocked in 5% (wt/vol) skim milk solution (Oxoid) (with 0.5% [vol/vol] polyoxyethylene-sorbitan monolaurate [Tween 20]) (Sigma) in phosphate-buffered saline (PBS) for 1 h at room temperature. To detect His-tagged proteins, a mouse His tag antiserum (Novagen) was used as a primary antibody at a 1:1,000 dilution in blocking solution overnight at 4°C. Following washing (in 1× PBS and 0.5% Tween 20), the secondary antibody (polyvalent peroxidase conjugate-coupled goat anti-mouse antibody; Sigma) at a 1:5,000 dilution in PBS–0.5% Tween 20 was applied for 1 h at room temperature. The membrane was then washed in 1× PBS and 0.5% Tween 20. A rabbit anti-FLAG antibody (Sigma) at a 1:1,000 dilution was used for detection of the C-terminal FLAG tag. The secondary antibody used for detection was donkey horseradish peroxidase-linked whole anti-rabbit immunoglobulin (Amersham) at a 1:5,000 dilution. In both cases, detection was performed using ECL solutions 1 and 2 (Amersham) and exposure to X-ray film.

Microscopy. Motility analysis of *H. pylori* was carried out by direct observation of cells using phase-contrast microscopy. Electron microscopy was used to visualize the presence of flagellar filaments on bacterial cells. Twenty microliters of cell culture was spotted onto a Formvar-coated grid (Agar Scientific) and incubated at room temperature for 1 min. Excess culture was washed away with sterile water, and bacteria and their flagellar filaments were stained with 20 μl of 0.5% (wt/vol) phosphotungstic acid, pH 7.0, for 30 s. Grids were then visualized using a JEOL JEM-100S electron microscope.

TABLE 2. Oligonucleotide primers used in this study

Primer Name	Sequence 5′ to 3′
HP1575UF	AGCGGTCGACTCCATCAAATAGCTAAAGTG
HP1575DR	CAATTCCATGGGTGATGAAAACGATGCTC
HP1575REGKUF	CGTGGGATCCGTGTTTTTATGGCTC
HP1575REGKDR	TGTTGAATTCTAGAGCGGCGGCTTT
HP0770MF	TTTTCATCGATGCTATCATTGATAACATTCC
HP0770MR	AATGCCTGCAGGTAGGGATATTATGCCTTTC
FlhBREGKUF	TAAAGGATCCCAAGAACGCCAAAAAC
FlhBREGKDR	TGGAGAATTCTCGCGCTAGGGAGTTC
HP0770NDR	GTAATGAATTCAGTTAGTAACCACGACATTG
HP0770FCP	CAATGAATTCAGGAGGGTTACTATGCCCACCCATTAC
HP0770KpnIGR	TACAGGTACCTTAAAGAGGTTTAATGATC
HP1575FCP	CGCTGAATTCAGGAGGTATTAGATGAATAAAACC
HP1575KpnIGR	TACAGGTACCTCACTTGGACATTTGCACG
FlhBU	GCCGAAGCTTCATCATCATCATCATCATATGGCTGAAGAAGAAAAAACC
FlhBD	AAAAGTCGACAAGAGGTTTAATGATCTTTTGTTTTTGGCG
ChloroF	TATCGAATTCTAAGTAATTAAGGAGGATAAATGATGCAATTC
ChloroR	TTTTGGATCCCATCAGTGCGACCTCCTGGGATTTTATTTATTCAGCAAG
ProlinF	AATTTGGTACCCATTACGCCGTCGCTCTC
ProlinR	AATTTGGTACCGTTAGTAACCACGACATTGGC
HP0770EcoRI	TACAGAATTCTTAAAGAGGTTTAATGATC

Analysis of free-swimming *H. pylori* cells using a Hobson BacTracker. A BacTracker (Hobson Tracking Systems, Sheffield, United Kingdom) was used to determine the run speed, stopping, and tumbling characteristics of motile bacterial cultures. After 24 h of growth on brain heart infusion-fetal bovine serum medium, 9.6 μl of culture was placed onto a microscope slide and covered with a 22- by 22-mm coverslip to give a chamber depth of 20 μm. Cultures were observed using a 20× phase-contrast objective on a Nikon Labophot 2A microscope and were tracked immediately for 100 tracks. New slides were made in this way an additional 44 times, and the experiment was repeated on more than three different occasions for each set of strains. Real-time computer tracking was carried out using the Hobson BacTracker 50 Hz system set to the image conditions outlined previously (25).

Phylogenetic analysis of FlhB2 proteins. The predicted protein sequences from *flhB2* gene homologues were aligned using CLUSTALW (36). A representative set of 39 sequences from completed genome sequences was selected for phylogenetic analysis. The phylogenetic relationships among the proteins were estimated by the Bayesian method implemented in MrBayes (14). The analysis used the Jones, Taylor, and Thornton model of evolution with gamma distributed rates across sites, and 10 million generations with a burn-in of 10%; estimated sample sizes from Tracer (31) were 3,000 or greater for all estimated parameters.

RESULTS

Homologues of HP1575 are found in other species. Sequence analysis of the available *H. pylori* genomes revealed the presence of a putative gene (HP1575 in strain 26695 and *flhB2* in strain J99) encoding a protein with similarity (38% identity) to the predicted $FlhB_{CC}$ domain of the *H. pylori* FlhB protein (hereafter referred to as $HP0770_{CC}$ and HP0770, respectively) (Fig. 1A). Using HP1575 in BLAST searches against sequence databases of finished and unfinished microbial genomes (http://www.ncbi.nlm.nih.gov; http://www.tigr.org; http://www.sanger.ac.uk), putative homologues were identified in more than 70 bacterial species, which expands on those already noted by Pallen et al. (27), and include both gram-positive and gram-negative organisms (see Table S3 in the supplemental material). HP1575 homologues were found to be present only in bacteria also containing the *flhB* gene. However, considering complete genome sequences and surveying at the level of genera, only about 55% of bacteria with *flhB* also had an HP1575-like gene. Among these, there were several species of medical importance (e.g., *Pseudomonas aeruginosa*, *Treponema pallidum*, and *Bordetella pertussis*), bacteria involved in bioremediation (e.g., *Geobacter sulfurreducens* and *Nitrosomonas europeae*), and other, well-characterized, motile bacteria such as *Caulobacter crescentus* and *Bacillus subtilis*. The enteric model bacteria *E. coli* and *S. enterica* serovar Typhimurium, however, do not appear to possess HP1575 homologues. This group of proteins will be collectively referred to as FlhB2 in the remainder of this study.

The lengths of the FlhB2-like proteins range from 53 amino acids in *Thiobacillus denitrificans* to 123 in *Rhodospirillum rubrum* and 160 in *Bacillus clausii*. Most FlhB2 proteins are found to be in the range of 85 to 95 amino acids in length, with HP1575 being 90 amino acids in length. Protein alignments (Fig. 1B) revealed two absolutely conserved amino acids (Pro^{20} and Pro^{65} in *H. pylori*) and several others that are highly conserved. Similar or identical amino acids are clustered in three main regions (Fig. 1B), ranging from amino acids 7 to 12, 19 to 38, and 64 to 80 (in HP1575). The largest extent of variation is seen at both C-terminal and N-terminal ends, which accounts for the different lengths of the proteins. Variation at the C-terminal end is also common in FlhB homologues, which are also of various lengths. The amino acid stretches that are highly conserved are mainly nonpolar or hydrophobic in nature, suggesting that these regions might be essential for the function of the protein. Three organisms, *Bacillus halodurans*, *Exiguobacterium* sp. strain 255-15, and *Geobacillus kaustophilus*, appear to contain two divergent copies of the *flhB2* gene. Interestingly, in these bacteria one of the *flhB2* homologues is located immediately upstream of the putative *fliS* and flagellin genes.

H. pylori HP1575 is located immediately downstream of HP1574 (*ribC*) (6), a gene involved in riboflavin synthesis (2), and might be transcribed from the same promoter. It lies upstream of *abcC*, which Hendricks and Mobley report to have significant similarity to the *nikD* gene of *E. coli*, encoding the ATP binding subunit of a nickel ABC transporter (12). A comparison between the gene regions surrounding the *flhB2* gene in related organisms belonging to the *Campylobacterale* showed that *Helicobacter hepaticus* and *Wolinella succinogenes*, but not *C. jejuni*, also contain a *ribC* gene immediately upstream of *flhB2*. However, this arrangement is not conserved in other bacteria.

Phylogenetic analysis of FlhB2 proteins. *flhB2* homologues are found in bacteria belonging to widely divergent phylogenetic groups, including the five clades (α to ε) of *Proteobacteria*, the *Firmicutes* (A+T-rich gram-positive bacteria), and *Spirochaetes*. The phylogenetic relationship among 39 representa-

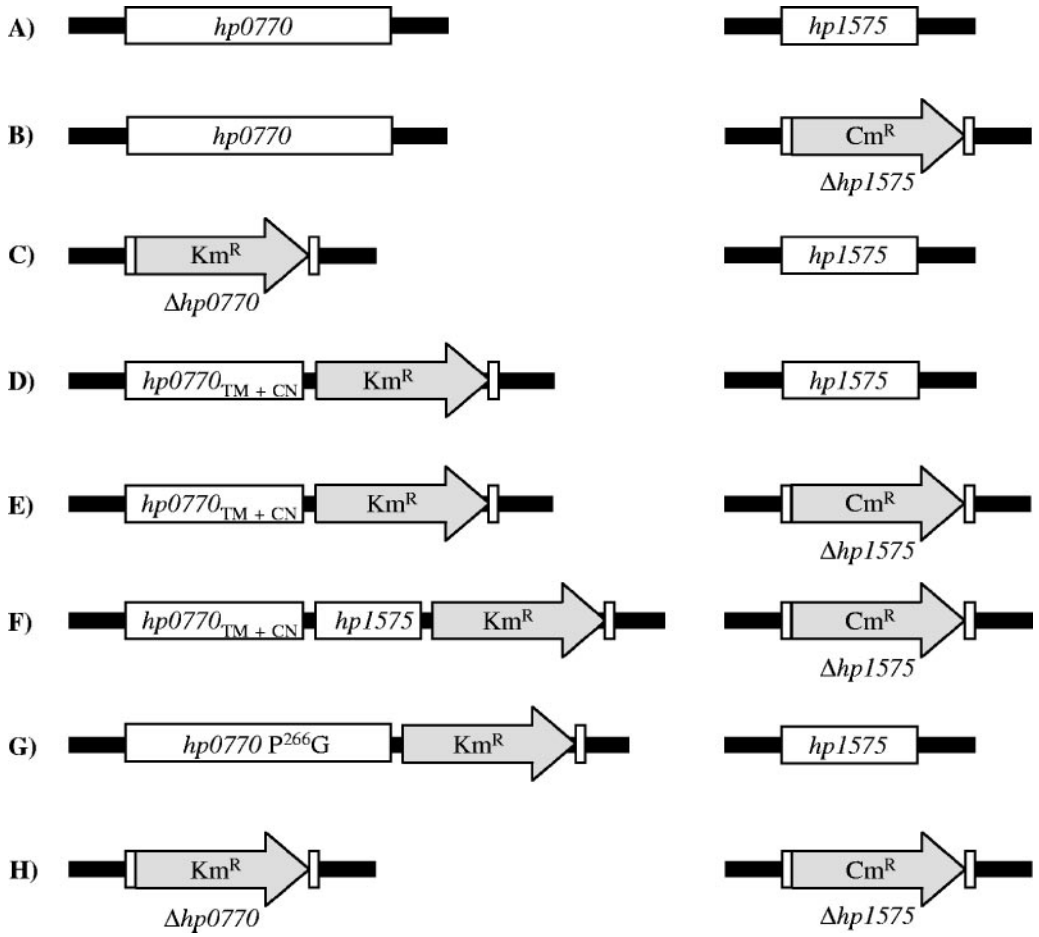

FIG. 2. Schematic representation of the gene regions of HP0770 and HP1575 mutants. The genetic elements comprising the HP0770 and HP1575 loci of strains J99ΔHP1575::Cm (B), J99ΔHP0770::Km (C), J99ΔHP0770$_{CC}$::Km (D), J99ΔHP0770$_{CC}$ΔHP1575 (E), J99HP0770$_{CC}$ΔHP1575HP1575 Comp (F), J99HP0770P^{266}G-Km (G), J99ΔHP0770ΔHP1575 (H), constructed in this study in comparison with the motile wild-type J99 (A) are shown. Gray arrows indicate kanamycin or chloramphenicol resistance genes; white boxes represent complete or truncated versions of HP0770 and HP1575.

tive sequences was estimated from a Bayesian analysis of protein sequences (Fig. 3). Despite the short length of the sequence alignment available (75 sites were used, after exclusion of gaps in the alignment), the tree showed many clades corresponding to the accepted relationships among these species as derived, for example, from analysis of ribosomal gene sequences. The *H. pylori* sequence was found to cluster with those from *H. hepaticus* and *W. succinogenes*, two other members of the ε-proteobacteria, in a strongly supported clade. This group of ε-proteobacteria formed a cluster with, but distant from, the clade of β-proteobacteria. These results provide no evidence for a recent acquisition of HP1575 by the *Helicobacter* lineage. In contrast, *C. jejuni*, the fourth member of the ε-proteobacteria included, did not fall within the ε-proteobacteria cluster, or even the larger clade including the β-proteobacteria, suggesting that the *C. jejuni* gene was acquired by horizontal gene transfer. In addition, *G. sulfurreducens* did not cluster with either of the other δ-proteobacteria in the analysis (*Desulfotalea psychrophila* and *Pelobacter carbinolicus*), but fell within the strongly supported clade otherwise comprised of β-proteobacteria, indicating a recent horizontal gene transfer.

Two of the species included in the phylogenetic analysis, *B. halodurans* and *Geobacillus kaustophilus*, have two *flhB2* homologues. One homologue from each species fell within the clade of *Firmicutes*, as expected. The second homologues from these two species were similar to each other but distant from the other firmicute sequences, grouping (albeit nonsignificantly) with the sequence from *Thermotoga maritima*. Thus, the second copy of *flhB* in these species does not appear to represent a recent gene duplication but, rather, an ancient horizontal gene transmission.

Deletion of HP0770 abolishes motility while HP1575 and HP0770$_{CC}$ mutants remain motile. In order to facilitate studies of the role of HP1575 and HP0770$_{CC}$ in flagellar assembly and motility, HP1575, HP0770$_{CC}$, and HP0770 deletions were constructed in *H. pylori* strains J99 and SS1. HP0770 and HP1575 were inactivated by deleting a large internal part of the respective gene and replacing it with either a kanamycin or a chloramphenicol resistance cassette (see Materials and Methods). Both cassettes lacked promoter and terminator sequences, thus ensuring that transcription of the disrupted gene was driven and terminated by the native promoter and terminator,

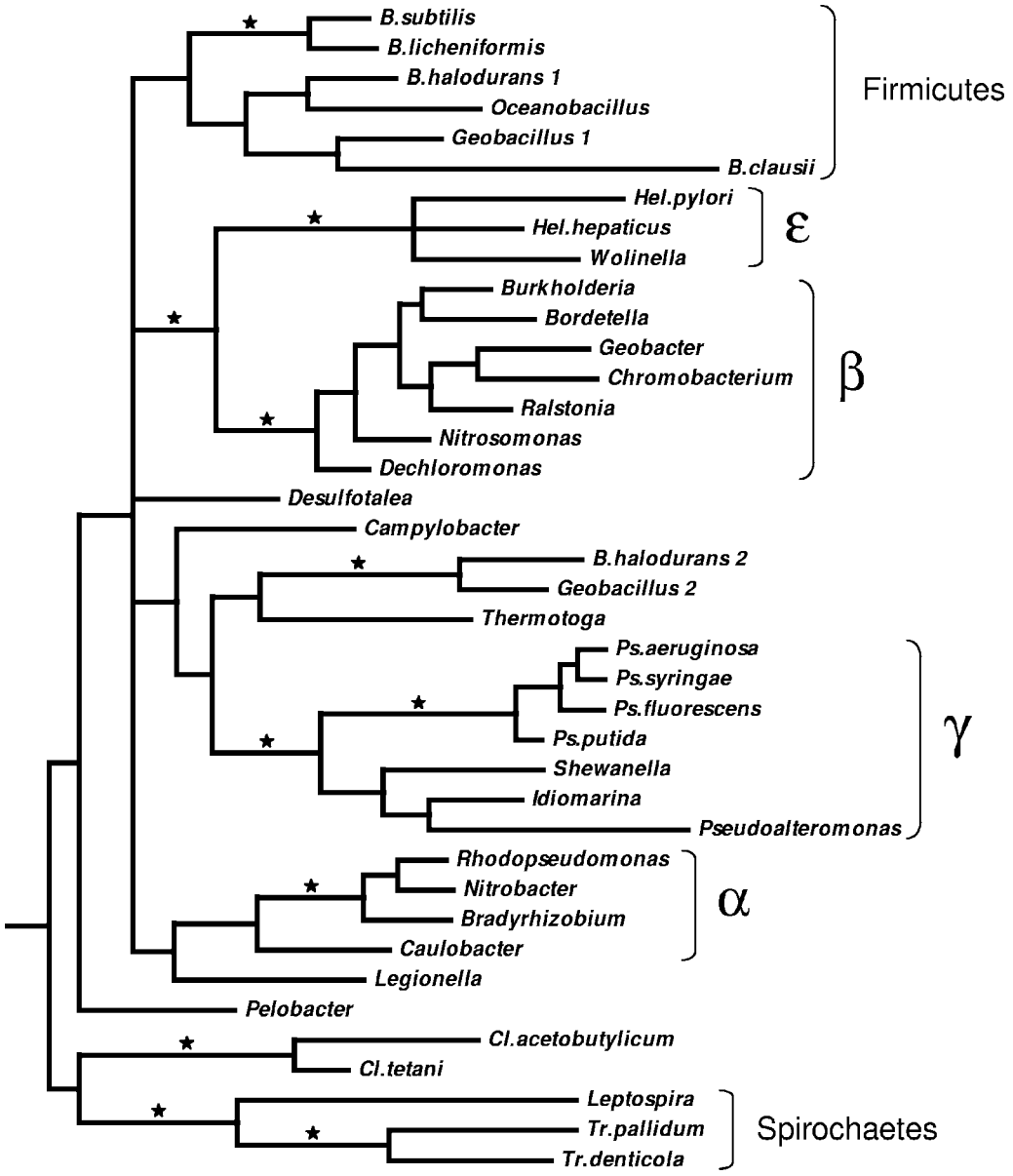

FIG. 3. Evolutionary relationships of FlhB2 homologues. Clades comprised of members of the α-, β-, γ-, and ε-*Proteobacteria*, as well as *Firmicutes* and *Spirochaetes*, are indicated by brackets at the right. Internal branches with posterior probabilities greater than 95% are indicated by asterisks. The abbreviated genus names are as described in the legend of Fig. 1. Full details of the species names and accession numbers for the sequences are available from the authors on request.

respectively. A J99 mutant expressing a truncated HP0770 protein lacking the $HP0770_{CC}$ domain (J99ΔHP0770$_{CC}$) was constructed by replacing the proline in the conserved NPTH motif of the processing site with a stop codon (TAG) immediately followed by the kanamycin cassette. In agreement with previous research (9) a complete HP0770 deletion abolished motility in *H. pylori*. However, a deletion of HP1575 or $HP0770_{CC}$ did not seem to affect motility in motility agar (Fig. 4). In agreement with these findings, electron microscopic images showed that no flagella were produced by the J99ΔHP0770::Km strain and that there was no obvious difference between flagellation of the wild-type J99 and the J99ΔHP1575::Km and J99ΔHP0770$_{CC}$::Km strains (Fig. 4). All results obtained for strain J99 were also observed with SS1, indicating that this result was not strain dependent (data not shown).

An *H. pylori* J99ΔHP0770$_{CC}$ΔHP1575 double mutant does not produce flagella and is nonmotile. The J99ΔHP0770::Km and J99ΔHP0770$_{CC}$::Km strains described above were used to generate double mutants also defective in HP1575. This was achieved by deleting the major part of the HP1575 gene in these strains and replacing it with a chloramphenicol resistance cassette. As expected, mutant strain J99ΔHP0770ΔHP1575 (with both HP0770 and HP1575 deleted) was nonmotile. Moreover, strain J99ΔHP0770$_{CC}$ΔHP1575 (no functional HP1575 but still producing a truncated HP0770) was, in contrast to its parent J99ΔHP0770$_{CC}$::Km, also nonmotile (Fig. 4).

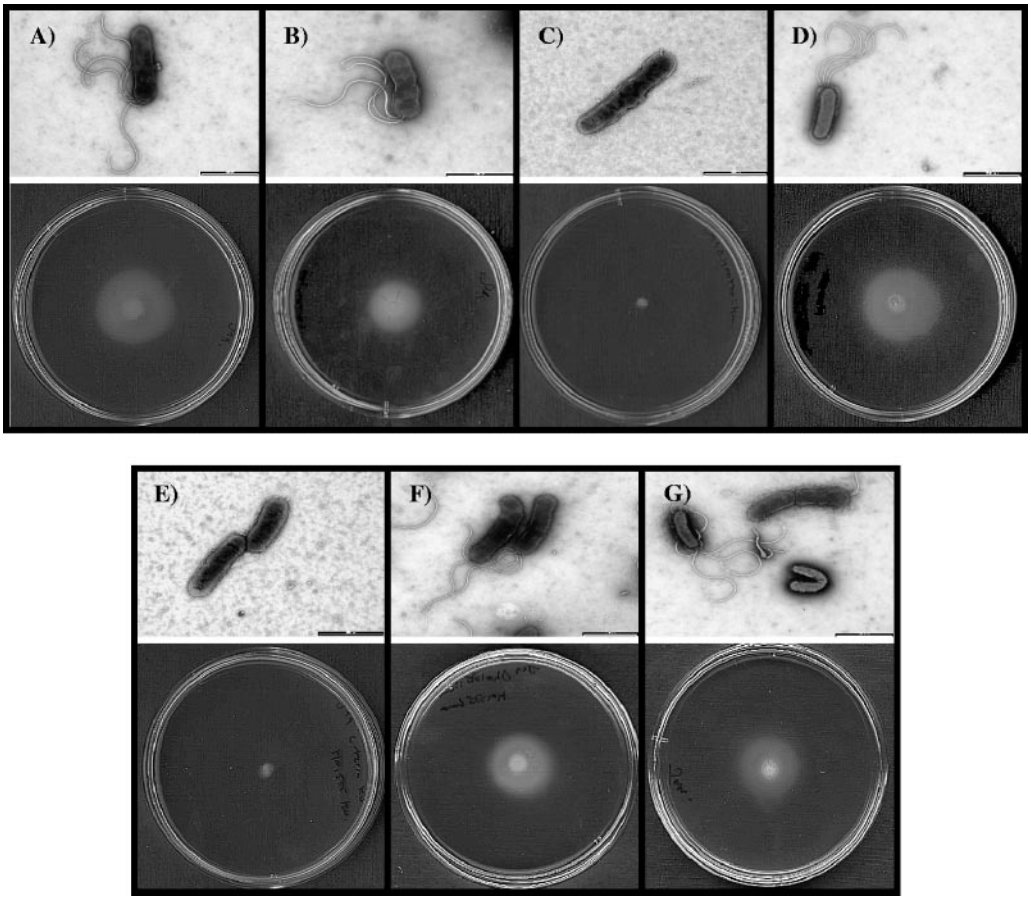

FIG. 4. Production of either HP0770$_{cc}$ or HP1575 is required for flagellum production and motility. Electron micrographs of negatively stained preparations of *H. pylori* cells (upper panel) and motility agar plates (lower panel) of strains J99 (A), J99ΔHP1575::Cm (B), J99ΔHP0770::Km (C), J99ΔHP0770$_{CC}$::Km (D), J99ΔHP0770$_{CC}$ΔHP1575 (E), J99ΔHP0770$_{CC}$ΔHP1575HP1575Comp (F), and J99HP0770(P266G)-Km (G) are shown. For the electron micrographs, the scale bar represents 2,000 nm. Wild-type motility and flagellum formation were observed when either HP1575 or HP0770$_{CC}$ was present in conjunction with HP0770$_{TM+CN}$.

Electron microscopy confirmed that both double mutants failed to produce flagellar filaments (Fig. 4). These data, in conjunction with the phenotypes of single mutants, suggested that either HP0770$_{CC}$ or HP1575 has to be present for flagellar protein export and, thus, filament assembly and motility to occur.

The reintroduction of HP1575 into J99ΔHP0770$_{CC}$ΔHP1575 restored motility and flagellation. Full-length HP1575 was reintroduced into the chromosome of the nonmotile *H. pylori* J99ΔHP0770$_{CC}$ΔHP1575 strain immediately downstream of, but not fused to, ΔHP0770$_{CC}$, with its own independent ribosome binding site, thus generating the complemented strain J99ΔHP0770$_{CC}$ΔHP1575HP1575Comp. Gene HP0770$_{CC}$ was introduced in the same manner as a control to create strain J99ΔHP0770$_{CC}$ΔHP1575HP0770$_{CC}$Comp. Both strains showed wild-type motility when tested in motility agar and produced flagellar filaments, lending strength to the proposal that either HP0770$_{CC}$ or HP1575 is required for flagellum assembly (Fig. 4).

HP0770 is processed in *E. coli*. HP0770, like many other putative FlhB homologues in the databases, contains the conserved NPTH motif which marks the predicted processing site of FlhB in *S. enterica* serovar Typhimurium (24). The conservation of this site suggests that proteolysis of FlhB is likely to be a general feature and is not confined to the few bacteria already analyzed experimentally (16, 24). To study whether HP0770 processing occurs in vivo, a modified HP0770 gene encoding an N-terminally His-tagged and C-terminally FLAG-tagged FlhB protein was constructed (see Materials and Methods). When this construct (plasmid pFLAGMW1) was heterologously overexpressed in *E. coli* DH5α, processing of the tagged HP0770 could be analyzed using antibodies directed against the respective tags (anti-His and anti-Flag, respectively). Under these conditions, both the processed and unprocessed forms of the protein were observed. The unprocessed form, which was recognized by both antibodies, had an apparent molecular mass of 42 kDa (Fig. 5). The putative HP0770$_{CC}$ domain had a mass of 12 kDa (Fig. 5B) and was only recognized by the anti-FLAG antibody, whereas the putative HP0770$_{TM+CN}$ had a mass of 31 kDa and was exclusively recognized by the anti-His antibody (Fig. 5A). The observed molecular masses for both cleavage products are in agreement with those predicted from the sequence when processing occurs at the NPTH site. A plasmid (pFLAGMW1A) containing

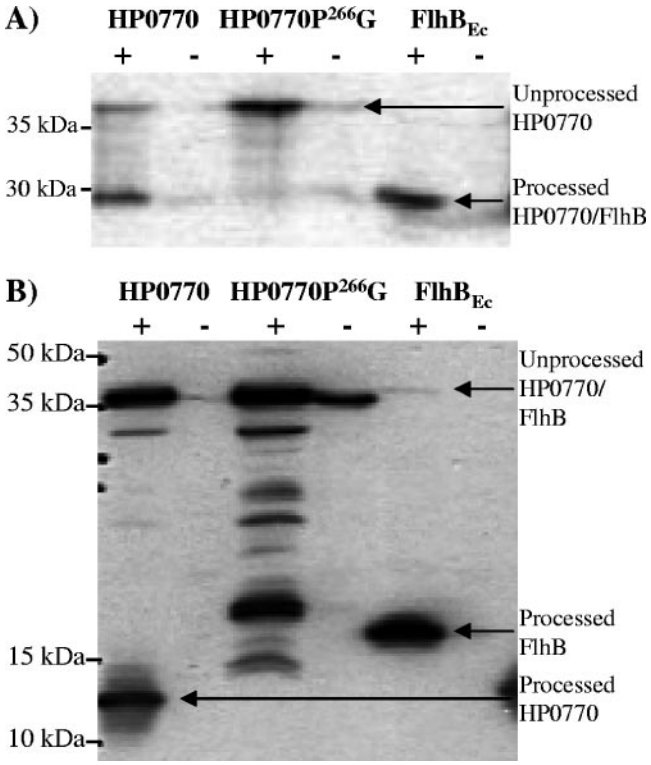

FIG. 5. HP0770(P266G) is not processed in the same manner as HP0770 in *E. coli*. Strains DH5α pFLAGMW1 (HP0770), DH5α pFLAGMW3 [HP0770(P266G)], and DH5α pFLAGMW1A (*E. coli flhB*) were grown in the presence (+) and absence (−) of isopropyl-β-D-thiogalactopyranoside. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and Western blotting of whole-cell extracts with either anti-HIS (A) or anti-FLAG (B) antibodies, it could be seen that HP0770 is processed at the predicted NPTH site and in an identical manner to *E. coli* FlhB. The C-terminal domain of *E. coli* FlhB is larger than its *H. pylori* counterpart, and this is reflected in the increased size of the protein detected by anti-FLAG. HP0770(P266G) produced increased amounts of unprocessed protein and a secondary protein of around 17 kDa. The latter could only be detected with anti-FLAG but not anti-HIS antibody, indicating that it contained the C terminus of HP0770.

similarly tagged *flhB* from *E. coli* ($flhB_{Ec}$) was constructed, and the cleavage of $FlhB_{Ec}$ was studied in *E. coli* DH5α (Fig. 5). A similar cleavage pattern was observed. The FLAG-tagged HP0770 gene was introduced into *H. pylori* on a suicide plasmid and replaced the wild-type gene on the chromosome. The resulting strain was motile (data not shown), showing (i) that the tagged protein was expressed in *H. pylori* and (ii) that the tags did not interfere with its function. However, neither the processed nor unprocessed form could be detected directly by Western blot analysis, probably due to low levels of HP0770 protein present in the cell (data not shown).

A mutation in Pro^{266} abolishes processing of HP0770 in *E. coli* but has no effect on motility. A point mutation that altered the Pro^{266} residue to Gly^{266} was introduced into the NPTH processing site of the tagged HP0770, thus creating the protein HP0770(P266G). *E. coli* DH5α cell extracts, expressing the tagged mutant gene (plasmid pFLAGMW3), were subjected to Western blot analysis using anti-His and anti-FLAG antibodies. The 12-kDa and 31-kDa cleavage products seen previously were absent. Instead, when HP0770 was probed with anti-FLAG, a secondary processing product of approximately 17 kDa was observed (Fig. 5B). When a FLAG-tagged version of HP0770(P266G) was introduced into the *H. pylori* chromosome in place of the native HP0770, the resultant strain [J99HP0770(P266G)-Km] showed parental motility. Furthermore, inactivation of HP1575 in this background [resulting in the double mutant J99HP0770(P266G)-Km ΔHP1575] had also no effect on motility (data not shown). This is different from the situation in *S. enterica* serovar Typhimurium, suggesting that if in vivo processing of wild-type HP0770 does occur in *H. pylori*, as is seen when heterologously expressed in *E. coli*, it is not essential for motility.

DISCUSSION

FlhB2 homologues are found in a wide range of bacteria. In this study we showed that homologues of HP1575, a protein with similarity to the C terminus of HP0770 (FlhB), are found in diverse range of motile bacteria. The phylogenetic relationships among these homologues, here designated FlhB2, are broadly consistent with the accepted classification of the species in which they are found (Fig. 3). There was some indication of horizontal transfer of *flhB2*, particularly for the genes found in *C. jejuni* and *G. sulfurreducens*. However, most of the other discordant branching positions were not strongly supported and may arise due to the high level of divergence among sequences and the short length of the alignment used. The tree provided no evidence to suggest that *H. pylori* acquired HP1575 through recent horizontal gene transfer, since it clustered with homologues from other closely related ε-proteobacteria. In a combined phylogenetic analysis with $FlhB_{CC}$ sequences, the FlhB2 homologues formed a monophyletic group (not shown). The function of FlhB2 seems to be linked to that of FlhB, since the homologues have only been identified in bacteria that also have the *flhB* gene; a similar suggestion has recently been made by Pallen et al. (27). We conclude that the FlhB2 homologues are due to a single very ancient duplication of the 3′ end of the *flhB* gene and hypothesize that all are involved in flagellar assembly and possibly type III secretion. However, FlhB2 is not essential for motility, since only about half of the bacteria with the *flhB* gene also have *flhB2*.

HP0770 is required for flagellar synthesis and function in *H. pylori*. In agreement with previous data (9), an *H. pylori* strain with a chromosomal deletion of HP0770 was shown to be nonmotile. Similar nonmotile phenotypes exhibited by *flhB* mutants of several other bacteria, e.g., *S. enterica* serovar Typhimurium (15, 24) and *C. jejuni* (19), indicate a universally conserved and essential function of FlhB in flagellum assembly. The complete loss of motility and flagellar filament assembly is probably due to a complete block in flagellum biogenesis, similar to that seen in *S. enterica* serovar Typhimurium, where only MS ring complexes are assembled (15). This is in agreement with the absence of flagellar filaments when ΔHP0770 strains were viewed by electron microscopy.

The presence of either $HP0770_{CC}$ or HP1575 is required for flagellar synthesis and, hence, motility to occur. Under conditions where full-length HP0770 is expressed, HP1575 is not required for the assembly of fully functional flagella. This initially suggested that the gene may not have a motility-related

function. However, a strain producing a truncated HP0770 protein lacking the $HP0770_{CC}$ domain also showed flagellum formation and wild-type motility, which gave the first indication that *H. pylori* might be able to compensate for the inactivation of $HP0770_{CC}$ with the production of HP1575. This is in contrast to *S. enterica* serovar Typhimurium, a bacterium that has no FlhB2 homologue, where loss of $FlhB_{CC}$ leads to incomplete flagellar assembly and, thus, nonmotility (10, 24). Comparisons between flagellation and motility in wild-type *H. pylori* and its respective ΔHP1575 and $\Delta HP0770_{CC}$ strains showed that there was little, if any, difference. All of them were motile, produced normal run-tumble motility (as viewed by the Hobson tracker) (data not shown), and produced wild-type-length flagellar filaments when viewed through the electron microscope. However, a role for HP1575 in flagellar assembly was revealed through the phenotype of a double deletion strain, $J99\Delta HP0770_{CC}\Delta HP1575$, which was nonflagellate and, thus, nonmotile. Importantly, both flagellum formation and motility were restored in complementation strains with either HP1575 or $HP0770_{CC}$ present immediately downstream of $\Delta HP0770_{CC}$. This confirmed the hypothesis that HP1575 protein can substitute for processed $HP0770_{CC}$, acting as a "spare part" for the *H. pylori* flagellar export apparatus. It is not clear, however, why so many motile bacteria contain an FlhB2 homologue in addition to FlhB. At least in *H. pylori* and under laboratory conditions, this protein is not essential for motility. We speculate that under certain conditions an additional $HP0770_{CC}$-like domain may be required for optimal flagellum formation in *H. pylori* to occur. For instance, an independently transcribed "copy" could serve to increase the ratio between $HP0770_{TM+CN}$- and $HP0770_{CC}$-like domains, which may influence timing and efficiency of flagellar assembly.

Processing of HP0770 is not essential for motility. In *S. enterica* serovar Typhimurium, mutations in the NPTH processing site render the bacterium nonmotile (24). Cleavage of FlhB is required for a FliK-driven conformational change, resulting in export substrate specificity switching, and failure to cleave FlhB gives rise to polyhooks (8). This is not true for *H. pylori*, where an equivalent point mutation that changed the conserved Pro^{266} to Gly had no observable effect on motility and did not result in polyhook formation. It is presently not known what is responsible for FlhB processing in any bacterium, although the process is possibly autocatalytic (8, 10). An alignment of the region surrounding the predicted primary processing site from all presently available FlhB homologues (data not shown) indicated that there are several conserved amino acids present around this site but gave no indication which of these may be needed for processing. Unfortunately, processing of HP0770, although occurring in *E. coli*, could not be demonstrated in *H. pylori* using antibodies directed against tagged HP0770. This is possibly due to a low copy number of HP0770. Similarly, YscU processing could only be detected by Western blot analysis in *Y. pseudotuberculosis* after the gene was overexpressed (16). In *Y. pseudotuberculosis* cleavage of YscU was abolished entirely when the NPTH site was mutated ($N^{263}A$, $P^{264}A$, or $T^{265}A$), but its function in Yop secretion was retained, indicating that YscU cleavage is not essential for Yop secretion (16). This is similar to *H. pylori* in that prevention of cleavage at the NPTH site did not affect the function of HP0770. For *H. pylori*, however, it is possible that secondary-site processing (as observed after heterologous overexpression of HP0770 in *E. coli*) compensated for the loss of cleavage at the NPTH site. The possibility that processing is not essential as long as an $FlhB_{CC}$ equivalent domain is provided in the form of HP1575 could be excluded: inactivation of HP1575 in the processing-defective background (resulting in the double mutant J99HP0770(P266G)-Km ΔHP1575) had no effect on motility.

In summary, we have shown that inactivation of HP1575 in *H. pylori* wild-type strains did not result in motility defects or other obvious phenotypes, suggesting that the gene may be redundant or that its effects are subtle. However, HP1575 can fully replace the $HP0770_{CC}$ domain in the flagellar export apparatus, suggesting that it is involved in flagellar biogenesis, at least under certain conditions. This may also be true for other members of the FlhB2 family. Whether HP1575 is differentially expressed under a subset of conditions encountered by *H. pylori* remains to be determined. It may only be switched on when required and then serve as a substitute for the $HP0770_{CC}$ domain. The protein may provide another form of regulation of flagellar assembly in *H. pylori*, an organism seen as lacking many other flagellar assembly checkpoints (26).

ACKNOWLEDGMENTS

We thank Karen Morehouse and Laura Hobley for assistance with BacTracker analysis and Liz Bailes for assistance with the phylogenetic analysis.

This work is supported by Ph.D. studentships for M.E.W. and K.J.E. provided by the Biotechnology and Biological Sciences Research Council, United Kingdom.

REFERENCES

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176–180.
2. **Bacher, A., M. Fischer, K. Kis, K. Kugelbrey, S. Mortl, J. Scheuring, S. Weinkauf, S. Eberhardt, K. Schmidt-Base, R. Huber, K. Ritsert, M. Cushman, and R. Ladenstein.** 1996. Biosynthesis of riboflavin: structure and mechanism of lumazine. Biochem. Soc. Trans. **24:**89–94.
3. **Danon, S. J., and K. A. Eaton.** 1998. Isogenic flagella mutants of *Helicobacter pylori*: candidates for an attenuated vaccine? Gut **43:**A38.
4. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. Med. Microbiol. **37:**123–127.
5. **Fan, F., K. Ohnishi, N. R. Francis, and R. M. Macnab.** 1997. The FliP and FliR proteins of *Salmonella typhimurium*, putative components of the type III flagellar export apparatus, are located in the flagellar basal body. Mol. Microbiol. **26:**1035–1046.
6. **Fassbinder, F., M. Kist, and S. Bereswill.** 2000. Structural and functional analysis of the riboflavin synthesis genes encoding GTP cyclohydrolase II (*ribA*), DHSP synthase (*ribBA*), riboflavin synthase (*ribC*), and riboflavin deaminase/reductase (*ribD*) from *Helicobacter pylori* strain P1. FEMS Microbiol. Lett. **191:**191–197.
7. **Fauconnier, A., A. Allaoui, A. Campos, A. Van Elsen, G. R. Cornelis, and A. Bollen.** 1997. Flagellar *flhA*, flhB and *flhE* genes, organized in an operon, cluster upstream from the *inv* locus in *Yersinia enterocolitica*. Microbiology **143:**3461–3471.
8. **Ferris, H. U., Y. Furukawa, T. Minamino, M. B. Kroetz, M. Kihara, K. Namba, and R. M. Macnab.** 2005. FlhB regulates ordered export of flagellar components via autocleavage mechanism. J. Biol. Chem. **280:**41236–41242.
9. **Foynes, S., N. Dorrell, S. J. Ward, Z. W. Zhang, A. A. McColm, M. J. G. Farthing, and B. W. Wren.** 1999. Functional analysis of the roles of FliQ and FlhB in flagellar expression in *Helicobacter pylori*. FEMS Microbiol. Lett. **174:**33–39.
10. **Fraser, G. M., T. Hirano, H. U. Ferris, L. L. Devgan, M. Kihara, and R. M. Macnab.** 2003. Substrate specificity of type III flagellar protein export in *Salmonella* is controlled by subdomain interactions in FlhB. Mol. Microbiol. **48:**1043–1047.

11. **Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch.** 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. J. Med. Microbiol. **38:**371–377.
12. **Hendricks, J. K., and H. L. T. Mobley.** 1997. *Helicobacter pylori* ABC transporter: effect of allelic exchange mutagenesis on urease activity. J. Bacteriol. **179:**5892–5902.
13. **Hirano, T., S. Yamaguchi, K. Oosawa, and S. Aizawa.** 1994. Roles of FliK and FlhB in determination of flagellar hook length in *Salmonella typhimurium*. J. Bacteriol. **176:**5439–5449.
14. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics **17:**754–755.
15. **Koroyasu, S., M. Yamazato, T. Hirano, and S.-I. Aizawa.** 1996. Kinetic analysis of the growth rate of the flagellar hook in *Salmonella typhimurium* by the population balance method. Biophys. J. **74:**436–443.
16. **Lavander, M., L. Sundberg, P. J. Edqvist, S. A. Lloyd, H. Wolf-Watz, and Å. Forsberg.** 2002. Proteolytic cleavage of the FlhB homologue YscU of *Yersinia pseudotuberculosis* is essential for bacterial survival but not for type III secretion. J. Bacteriol. **184:**4500–4509.
17. **Macnab, R. M.** 1996. Flagella and motility, p. 123–145. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schacchter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
18. **Marshall, B.** 2002. *Helicobacter pylori*: 20 years on. Clin. Med. **2:**147–152.
19. **Matz, C., A. H. van Vliet, J. M. Ketley, and C. W. Penn.** 2002. Mutational and transcriptional analysis of the *Campylobacter jejuni* flagellar biosynthesis gene *flhB*. Microbiology **148:**1679–1685.
20. **Ménard, R., P. J. Sonsonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899–5906.
21. **Minamino, T., T. Iino, and K. Kutsukake.** 1994. Molecular characterisation of the *Salmonella typhimurium flhB* operon and its protein products. J. Bacteriol. **176:**7630–7637.
22. **Minamino, T., and R. M. Macnab.** 1999. Components of the *Salmonella* flagellar export apparatus and classification of export substrates. J. Bacteriol. **181:**1388–1394.
23. **Minamino, T., and R. M. Macnab.** 2000. Interactions among components of the *Salmonella* flagellar export apparatus and its substrates. Mol. Microbiol. **35:**1052–1064.
24. **Minamino, T., and R. M. Macnab.** 2000. Domain structure of *Salmonella* FlhB, a flagellar export component responsible for substrate specificity switching. J. Bacteriol. **182:**4906–4914.
25. **Morehouse, K. A., I. G. Goodfellow, and R. E. Sockett.** 2005. A chimeric N-terminal *Escherichia coli*-C-Terminal *Rhodobacter sphaeroides* FliG rotor protein supports bidirectional *E. coli* flagellar rotation and chemotaxis. J. Bacteriol. **187:**1695–1701.
26. **Niehus, E., H. Gressmann, Y. Fang, R. Schlapbach, M. Dehio, C. Dehio, A. Stack, T. F. Meyer, S. Suerbaum, and C. Josenhans.** 2004. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori*. Mol. Microbiol. **42:**947–961.
27. **Pallen, M. J., C. W. Penn, and R. R. Chaudhuri.** 2005. Bacterial flagellar diversity in the post-genomic era. Trends Microbiol. **13:**143–149.
28. **Parkinson, J. S.** 1978. Complementation analysis and deletion mapping of *Escherichia coli* mutants defective in chemotaxis. J. Bacteriol. **135:**45–53.
29. **Penn, C. W., and C. J. Luke.** 1992. Bacterial flagellar diversity and significance in pathogenesis. FEMS Microbiol. Lett. **100:**331–336.
30. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
31. **Rambaut, A., and A. J. Drummond.** 2003. Tracer, version 1.2. [Online.] http://evolve.zoo.ox.ac.uk/.
32. **Ryan, K. A., Q. N. Karim, M. Worku, C. W. Penn, and P. W. O'Toole.** 2005. *Helicobacter pylori* flagellar hook-filament transition is controlled by a FliK functional homolog encoded by the gene HP0906. J. Bacteriol. **187:**5742–5750.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Segura, A., E. Duque, A. Hurtado, and J. L. Ramos.** 2001. Mutations in genes involved in the flagellar export apparatus of the solvent-tolerant *Pseudomonas putida* DOT-T1E strain impair motility and lead to hypersensitivity to toluene shocks. J. Bacteriol. **183:**4127–4133.
35. **Suerbaum, S., and P. Michetti.** 2002. *Helicobacter pylori* infection. N. Engl. J. Med. **347:**1175–1186.
36. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence-weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
37. **Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. C. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenny, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karpi, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:** 539–547.
38. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. *In vivo* transfer of genetic information between gram-positive and gram-negative bacteria. EMBO J. **4:**3583–3587.
39. **Van Arnam, J. S., J. L. McMurry, M. Kihara, and R. M. Macnab.** 2004. Analysis of an engineered *Salmonella* flagellar fusion protein, FliR-FlhB. J. Bacteriol. **186:**2495–2498.
40. **Wang, Y., and D. E. Taylor.** 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. Gene **94:**23–28.
41. **Williams, A. W., S. Yamaguchi, F. Togashi, S. I. Aizawa, I. Kawagishi, and R. M. Macnab.** 1996. Mutations in *fliK* and *flhB* affecting flagellar hook and filament assembly in *Salmonella typhimurium*. J. Bacteriol. **178:**2960–2970.
42. **Yanish-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–109.